IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of Tae-Yoon Kim et al. Application No.: 10/589,102 Filed: October 05, 2007 For: THERAPEUTIC USE OF CPG OLIGODEOXYNUCLEOTIDE FOR SKIN DISEASE	Group Art Unit: 1632 Examiner: NOBLE, MARCIA STEPHENS Confirmation No.: 5838

DECLARATION OF DR. Tae-Yoon Kim

Sir:

1. I, Dr. Tae-Yoon Kim, declare the following:

2. I am a citizen of the Republic of Korea, and have the following mailing address: Asia Seonsoochon Apt. #8-702, Jamsil-dong, Songpa-gu, Seoul 138-220, Republic of Korea;

3. I graduated from the Catholic University of Korea, Medical College with M.D. degree in 1980 and Ph.D. degree in 1989;

4. I am a professor of the Department of Dermatology, Catholic University of Korea;

5. I have read and am familiar with the above-identified United States patent application filed April 28, 2006, and I am submitting this Declaration in support of that application;

6. I have performed and/or supervised the experiments reported below

<Materials and Method>

<Effect of CpG-ODN 46-O via intravenous injection >

1> Preventive effect on the hyperimmune response of CpG-ODN 46-O via intravenous injection in the skin hyperimmune response model induced by the ovalbumin antigen

We investigated the preventive effect on atopic dermatitis of CpG-ODN by pre-injecting CpG-ODN intravenously and inducing hyperimmune response in skin with ovalbumin(OVA) in Balb/c mouse.

An antigen-specific atopic dermatitis model was prepared as shown in Fig. 1-1. Balb/c mice were primed by i.p.(intraperitoneal) injection with OVA antigen and alum (aluminum hydroxide), which is an adjuvant enhancing immune response, three times at one week intervals. To evoke a skin immune response, mice were sensitized with bandages applied with antigen or phosphate buffered saline(PBS, control) by attaching the bandages onto the back of the mice.

The mice were divided into three groups; Group 1 was intravenously injected 20μg/100μl of CpG-ODN on Day 0, Group 2 was intravenously injected 20μg/100μl of CpG-ODN on Day 0 and Day 21, and Group 3 was intravenously injected 20μg/100μl of CpG-ODN on Day 0 Day 7, Day 14 and Day 21. The CpG-ODN injected mice listed above were primed by i.p injection of 10μg of OVA protein mixed with 4mg of aluminum hydroxide on Day 8, Day 15 and Day 22. From Day 28, 100 μg of OVA in 100 μl PBS was applied to the shaved back of each mouse by a 1cm x 1cm patch of sterile gauze, and immune response was induced for 7 days. A week following Day 42, immune response was induced once again with an OVA patch. As a negative control of this test, PBS was injected in every injection, and as a positive control of this test, PBS was injected alone at the time of the CpG-ODN injection.

To investigate whether CpG-ODN 46-O can prevent the occurrence of the lesion induced by the above-mentioned methods, the test reagent (CpG-ODN (46-O)), a

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positive reagent (1826-S), and a negative control (PBS) were intravenously injected once, two times, and four times, respectively, prior to antigen injection. And, after priming, the skin, serum and spleen were extracted from the mouse to investigate the preventive effects thereof.

As a result, as shown in Fig. 1-2A, the skin lesion of the mouse treated with the test reagent and OVA by i.v. injection showed remarkable improvement compared with that of the mouse treated with PBS. In addition, the test reagent proved to be more effective as the frequency of injections is increased.

Fig. 1-2B demonstrates the preventive effects of the test reagent on atopic dermatitis by extracting antigen-exposed skins from mice and staining them with hematoxylin and eosin (H&E). Hyperkeratosis and acanthosis resulted in remarkable decrease in skin lesions of the mice which were intravenously injected with the test reagent, CpG-ODN 46-O, and positive reagent, 1826-S. The mice in Group 3, which were injected with 20μg/100μl of CpG-ODN four times, were cured like the mice which rarely have skin lesions.

In addition, the production level of IgE and IgG1, which are markers of the Th2-type immune response and IgG2a, which is marker of the Th1-type immune response in serum were investigated using ELISA. We compared the production level of IgE and IgG1 in the positive control group (receiving the OVA only) and the experimental group (Group 1 to 3 for the test reagent ,CpG ODN 46-O, and positive reagent, 1826-s). The results indicated that the mice of the experimental group showed remarkably low levels of IgE and IgG1, and more frequently CpG-ODN were injected, the lower levels of IgE and IgG1 were observed. In addition, the production level of the antigen specific IgG2a was increased by CpG-ODN. These results demonstrate that when CpG-ODN is injected in advance for prevention, it may inhibit the generation of Th2 type antibodies (Fig. 1-3).

IL-4 is a typical cytokine which increases Th2 type antibodies, such as IgG1 and IgE. Increased IL-4 stimulates the B cell to produce IgE and IgG1. It has been reported that CpG-ODN binds to the TLR9 of dendritic cells and increases Type I

IFN, thereby affecting Th1 type differentiation of primary T cells. However, it has also been reported that CpG-ODN is unable to differentiate already-differentiated T cells. Therefore we investigate the effect of CpG-ODN 46-O on IL-4 level. We isolated cells from the lymph nodes of the mouse on 48th day, cultured them with OVA, and investigated the level of IL-4. As shown in Fig. 1-4, the level of IL-4 decreased remarkably even when they were once again treated with OVA. This result indicates that pretreatment with the CpG-ODN of the present invention prevents differentiations into Th2-type immune cells and also demonstrates the preventive effect of the CpG-ODN on the present invention for Th2-type immune responses.

2> Treatment effect of CpG-ODN 46-O on the skin hyperimmune response model induced by ovalbumin

The hyperimmune response model was prepared using Balb/c mice. The ovalbumin antigen was applied to the skin of Balb/c mice, thereby inducing lesions CpG-ODN was intravenously injected into the lesions of the mice and the cureness of atopic dermatitis was investigated.

As shown in Fig. 2-1, to prepare the antigen specific atopic dermatitis animal model, Balb/c mice were primed by i.p. with OVA (antigen) and alum (adjuvant) three times at one week intervals. In order to induce skin immune response, OVA and PBS as a control were placed on the back of the mice for one week. Mice were sensitized with bandages to which OVA or PBS (control) had been applied and which were attached to the back of the mice for one week. To investigate the treatment effect on lesions of the mouse, CpG-ODN 46-O and PBS (control) were intravenously injected for 5 days. To confirm whether the skin immune response to antigens could be induced again, mice were once more sensitized with bandages containing antigen or PBS applied by attaching the bandages onto the backs of the mice for one week. The skin, serum and spleens of the mice were extracted to investigate the treatment effect thereupon.

As shown in Fig. 2-2A, skin priming by OVA caused skin lesions, and it was confirmed with even the naked eyes that the skin lesions remarkably improved with

the treatment of CpG-ODN by comparing the lesions of PBS treated mice(Ova+PBS) and CpG-ODN treated mice(Ova+46-O). The results of H&E staining of the extracted skin are shown in Fig. 2-2. It was confirmed that hyperkeratosis and acanthosis decreased remarkably (x200) in the lesions of Ova+46-O mice.

In addition, we investigated the levels of total IgE and antigen specific IgE in the serum of the mouse group mentioned above, using ELISA. As shown in Fig. 2-3, the levels of total IgE and antigen specific IgE increased, while antigen levels decreased markedly by the treatment with CpG-ODN.

Next, we investigated cytokine IL-4, which induced the expression of IgE and IgG1. Cells were isolated from the lymph nodes of each mouse and co-cultured with CpG-ODN. The level of IL-4 was measured by the method described in Fig. 1-4. As shown in Fig. 2-4, cells which were isolated from a CpG-ODN treated mouse did not express IL-4 at all, even when treated with OVA again. This result suggests that once skin hypersensitive response is treated by CpG-ODN, re-exposure to that antigen would not induce another skin hypersensitive reaction for a long time. Therefore, this is an example showing that CpG-ODN has long term effectiveness. In addition, the level of IL-4 of isolated lymphocytes from the non-treated mouse, co-cultured with ovalbumin and CpG-ODN, was remarkably lower than that of cells treated only with ovalbumin.

These results demonstrate that CpG-ODN 46-O can effectively treat atopic dermatitis induced by ovalbumin.

< The Effect of CpG-ODN O-46 by intraperitoneal injection>

3> Atopic dermatitis animal model induced by hapten (TNCB) in NC/Nga mouse and treatment Effect

In a NC/Nga mouse, atopic dermatitis occurs from the 8th week after birth in general circumstance, not in aseptic circumstance, however in SPF (specific

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pathogen free) condition, atopic dermatitis does not occur. Therefore, we investigated treating processes by inducing atopic dermatitis by application of TNCB to the skin and injecting CpG-ODN by i.p in SPF condition.

As shown in Fig. 3-1, we induced atopic dermatitis in the skin of a mouse by applying TNCB for 6 weeks and then injected test reagent, CpG-ODN by i.p. once a day for five consecutive days. After three days, the serum and skin were sampled and investigated whether atopic dermatitis was treated or not. Each group consisted of five mice.

As shown in Fig. 3-2A, the skin lesions of the mouse injected with CpG-ODN (46-O) by i.p. was remarkably treated when compared with that of the control (PBS-treated) group. In addition, H&E staining of the skin lesion sites showed that hyperkeratosis and acanthosis were lessened in the lesions of the mouse injected with CpG-ODN (46-O) as shown in Fig. 3-2B (x200).

In addition, the level of IgE in the serum of the NC/Nga mouse suffering from atopic dermatitis induced by TNCB and by injected i.p with CpG-ODN (46-O), was examined using ELISA. As shown in Fig. 3-3, the level of IgE remarkably decreased in the 46-O treated mouse.

4> Effect of CpG-ODN 46-O on delayed immune suppressive response by TMA priming

We investigated whether immune suppression in a mouse caused by the successive administration of TMA (trimellitic anhydride) recovers by an ip injection with CpG-ODN.

8-week old female Balb/c mice (weighing about 20g in bodyweight) were divided into three groups: a negative control group that was not primed with TMA, a positive control group that was primed with TMA; and a experimental group that was primed with TMA and i p injected with CpG-ODN 46-O. Each group consisted of five Balb/c mice.

To induce a priming response of the mouse using TMA, we shaved the abdominal region of a female Balb/c mouse and applied a 100 ㎕ mixture of acetone and olive oil (4:1) dissolved in a 10% TMA solution (Trimellitic anhydride; Sigma-Aldrich, USA) to the region and primed. Immediately, 20 ㎍ of CpG-ODN 46-O dissolved in PBS with a concentration of 1 ㎎/㎖ was injected by i.p as the experimental group. For the negative control, a mixture of acetone and olive oil without TMA was used. For a positive control and negative control, only PBS was injected rather than CpG-ODN. The 5th day after priming, a second priming was performed, as the method described above. From the 5th day to the 8th day after second priming, we measured the thickness of the ear of each mouse and applied 12.5 ㎕ of a 10% TMA solution to both ears of the mouse to induce atopic dermatitis. After 24 hours, the edema of the ears was measured using a micrometer (Fig. 4-1).

As shown in Fig. 4-2, dermatitis was induced in the shaved abdominal region of the mouse by applying 100 ㎕ of a 10% TMA solution twice (negative control (PBS alone): 31.0± 1.6× 10^{-2} mm, positive control (TMP alone): 68.8± 0.7× 10^{-2} mm). Meanwhile CpG-ODN 46-O injection by i.p inhibited the increased immune response by TMA.

<Asthma model for intravenous injection>

5> Effect of CpG-ODN 46-O on Asthma model

Asthma is a type of hypersensitive allergic disease like atopic dermatitis. They are similar to each other in terms of IL-4 and IgE being secreted in excess. Therefore, we investigated whether or not CpG-ODN is effective on asthma.

As shown in Fig. 5-1, in order to induce immune response with the ovalbumin (OVA) antigen, a Balb/C mouse raised in PF conditions was primed by s.c.(subcutaneous injection) with 25㎍ of OVA and 1 mg of aluminum hydroxide on

Day 0 and 14. On Days 21, 22, and 23, immune response was induced by administrating 20 μg of OVA dissolved in 50 μl of PBS into the airway, while on Days 20, 21, 22 and 23, 20 μg/100 μl of 1826-s and 100 μg/100 μl of CpG-ODN were injected by i.v.(intravenous injection), respectively. As a negative control for this test, PBS was injected in all injections and as a positive control, PBS was injected only in the CpG-ODN injections. On the day after last injection, airway hyper-responsiveness was induced with methacholine and enhanced pause (Penh), which reflects mathematically calculated symptons of Bronchiolitis obliterans using plethysmography. In addition, bronchoalveolar lavage fluid (BALF) of the mouse was collected and eosinophiles, which accumulate during asthma, were counted and the levels of IgG1, IgG2a, and IgE in the serum were measured by ELISA. Thereafter, lung tissue was fixed and stained with H&E to investigate the proliferation of epithelial cells in the airway.

The results of enhanced pause (Penh) are shown in Fig. 5-2. It shows that symptoms of bronchiolitis obliterans of CpG-ODN treated group decreased compared to those of the positive control (see Fig. 5-2). Also, CpG-ODN remarkably inhibited cell proliferation (Fig. 5-3) and decreased the number of eosinophiles (Fig. 5-4) and the level of OVA-specific IgE (Fig. 5-5).

These results demonstrate that CpG-ODN has a preventive effect on asthma, which is one of the Th2 type diseases, as well as on atopic dermatitis. When CpG-ODN 46-O was administrated in high concentrations, it was also shown to have effects on asthma; thus, it may be used as a treatment reagent for patients who have asthma.

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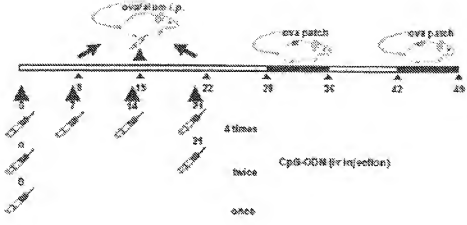
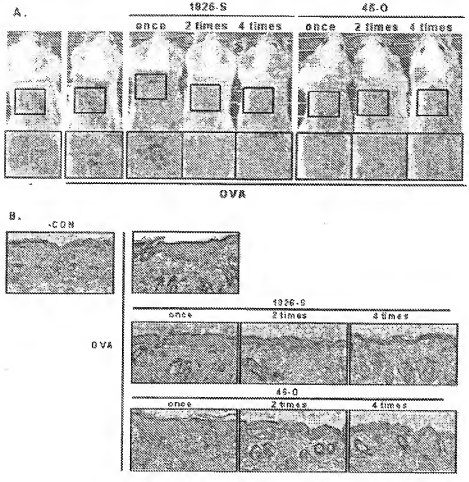
<Figures>

<Fig. 1-1>

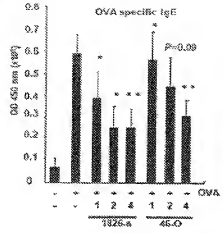


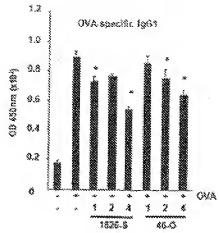
<Fig. 1-2>

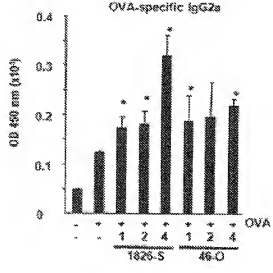

A.
1826-S
once
2 times
4 times
46-0
once
2 times
4 times
OVA
B.
-CON
OVA
1826-S
once
2 times
4 times
46-0
once
2 times
4 times

10

<Fig. 1-3>


OVA specific IgE
OD 450 nm (x10²)
P=0.09
OVA
1 2 4 1 2 4
1826-5
46-O


OVA specific IgG1
OD 450nm (x10²)
OVA
1 2 4 1 2 4
1826-5
46-O


OVA-specific IgG2a
OD 450 nm (x10²)
OVA
1 2 4 1 2 4
1826-5
46-O

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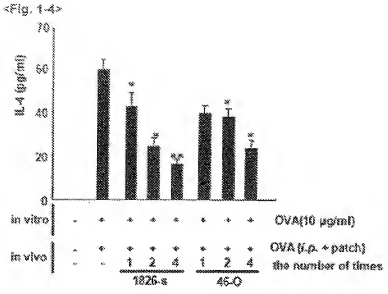
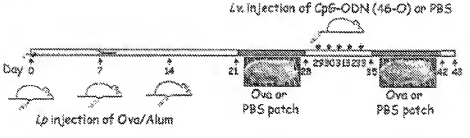
<Fig. 1-4>



<Fig. 2-1>



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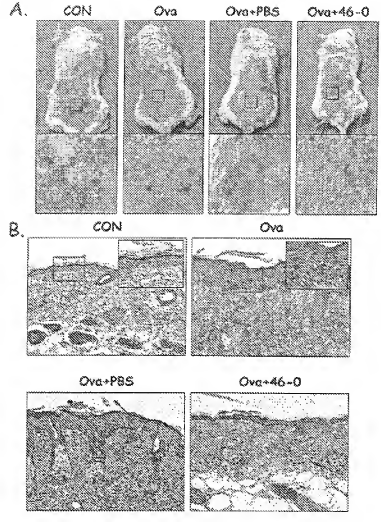
<Fig. 2-2>



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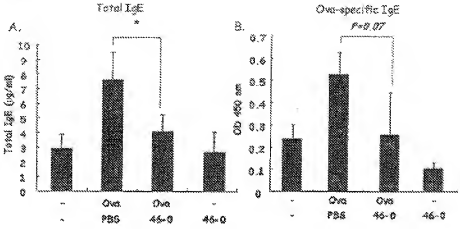
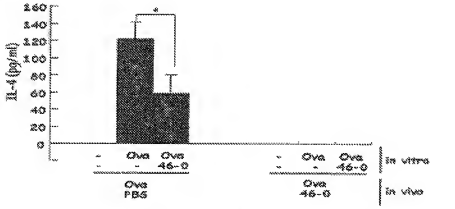
<Fig. 2-3>



<Fig. 2-4>



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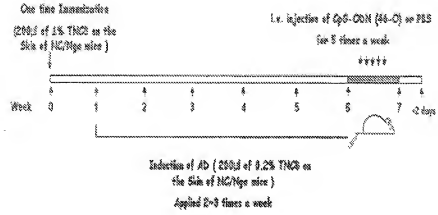
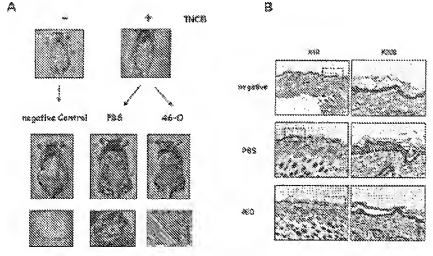
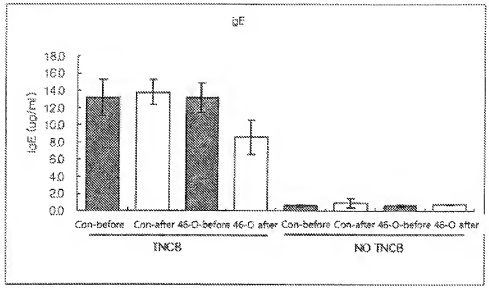
<Fig. 3-1>


One time Immunization
(200μl of 1% TNCB on the Skin of NC/Nga mice)
i.v. injection of CpG-ODN (46-O) or PBS
for 5 times a week
Week 0 1 2 3 4 5 6 7 +2 days
Induction of AD (200μl of 0.2% TNCB on the Skin of NC/Nga mice)
Applied 2~3 times a week

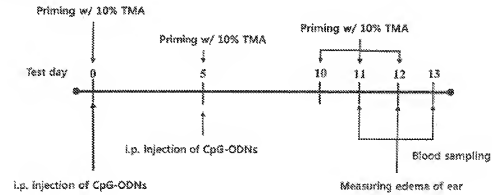
<Fig. 3-2>


A
-
+
TNCB
negative Control
PBS
46-O
B
X40
X200
negative
PBS
46O

<Fig. 3-3>



<Fig. 4-1>



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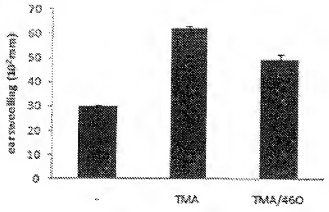
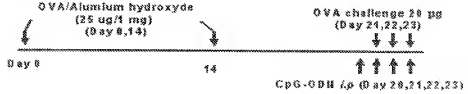
<Fig. 4-2>



<Fig. 5-1>



<Fig. 5-2>

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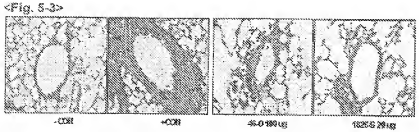
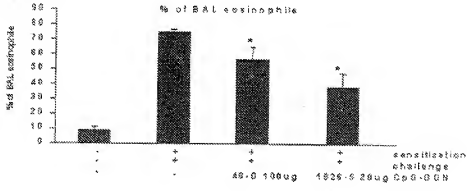
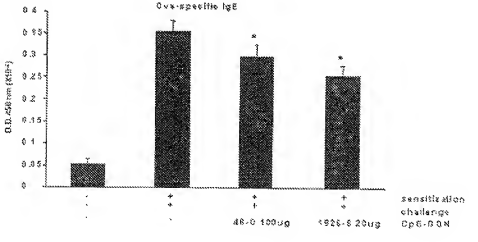
<Fig. 5-3>



<Fig. 5-4>



<Fig. 5-5>



7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date April 16, 2010 By _____
Tae-Yoon Kim